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**ECBC-TR-577**

# FORENSIC DISCRIMINATION OF 25 ISOLATES OF *BURKHOLDERIA MALLEI*

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In this study, the subspecies differentiation of 25 isolates of *Burkholderia mallei* was attempted based on their ribotype polymorphisms. The isolates were from human and equine infections that occurred at various times around the world. DNA samples from each isolate were digested with *Pst*I and *Eco*RI enzymes and probed with an *Escherichia coli*-derived 18-mer rDNA sequence to identify diagnostic fragments. Seventeen distinct ribotypes were identified from the combined data obtained with the two restriction enzymes. The results demonstrate the general utility of ribotyping for the subspecies identification of *B. mallei* isolates.

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# FORENSIC DISCRIMINATION OF 25 ISOLATES OF *BURKHOLDERIA MALLEI*

## 1. INTRODUCTION

In recent years, the scientific basis for the identification of microorganisms has undergone a shift in emphasis from the traditional reliance on biochemical and microscopic identification of phenotypic characteristics to techniques based on nucleotide sequence heterogeneities.[1] Some of these techniques have been used to distinguish strains at the subspecies level and thereby provide a sound basis for the epidemiological tracking of the likely source of an outbreak. These approaches typically rely on some variation of a DNA "fingerprint," a unique or diagnostic hybridization pattern arising from the amplification or probing of repetitive sequences occurring in polymorphic regions of the genome.

Ribotyping is one such fingerprinting approach. Bacterial ribosomal RNA (rRNA) operons comprise a family of highly conserved genes, each of which is flanked by regions of DNA with much greater variability than that encoding the rRNA operons themselves. Restriction fragment length polymorphisms (RFLPs) arising from sequence differences in the flanking restriction sites (or from insertions, deletions, or re-combinations within the rDNA-containing fragments) can be identified by probing restriction-digested, size-fractionated, and immobilized DNA fragments with labeled homologous DNA sequences. An advantage of ribotyping is that it enables genetic analysis of an organism without prior knowledge of its genomic DNA sequence. In addition, it can be a sensitive means to identify genetic heterogeneity in a readily interpretable pattern.

In the present work, the subspecies discrimination of 25 isolates of *B. mallei* was approached through polymorphisms identified by ribotyping, using *Pst*I and *Eco*RI restriction enzymes. Ribotyping was previously used by others[2-8] to characterize isolates of the related organism *Burkholderia pseudomallei*, the causative agent of melioidosis, which is a significant public health problem in Southeast Asia and Northern Australia. A total of at least 22 different ribotypes were described from *B. pseudomallei.*

There are no previously reported *B. mallei* DNA polymorphisms known to us. We believed the previous success with *B. pseudomallei* suggested the utility of ribotyping for subspecies discrimination of *B. mallei.*

*B. mallei* is a Gram-negative rod-shaped obligate parasite that causes Glanders primarily in equines, but also in humans. Cats, dogs, and many other mammals can be infected under experimental conditions, while hamsters[9] and mice[10] are the most common laboratory models with which to study *B. mallei*. Mortality is very high, there is no vaccine, and a chronic form of the disease sometimes develops that can exacerbate into the acute form even after many years.[11] Glanders has disappeared from most regions of the world, leaving only enzootic foci in Asia and eastern Mediterranean countries and sporadic human cases among those whose occupations involve direct contact with infected equines or work with the organism in laboratories. The organism has received increased attention recently because it was

designated by the U.S. Centers for Disease Control and Prevention as a Category B Bioterrorism Agent, (http://www.scchealth.org/docs/doche/bt/cats.html). Also, it has been reported recently that German saboteurs maliciously injected *B. mallei* into animals during World War I Other published reports include the construction of *B. mallei* strains containing multiple antibiotic resistance genes[14], a study of the correlation of antibiotic resistance with infectivity[15] and its alleged intentional release in Afghanistan.[16] These reports suggest the importance of developing a reliable means for the forensic discrimination of various isolates of the organism, which was the objective of this work.

## 2. MATERIALS AND METHODS

### 2.1 Sources and Growth of Bacteria.

Table 1 summarizes the available information on the strains used in this study.

### 2.2 DNA Isolation.

Isolates were streaked on Luria Broth (LB) plates supplemented with 4% glycerol and grown at 37 °C for 1-2 days. Individual colonies were inoculated into 5 mL LB + 4% glycerol liquid medium. Suspended cells (5 mL) were centrifuged at 5,000 x "g" for 15 min, and the resulting pellet was vigorously re-suspended and washed in 4 mL TS buffer (0.05 M NaCl, 0.02 M Tris, pH 8). Vigorous re-suspension was apparently critical to obtain digestible DNA and was presumably related to the removal of the polysaccharide capsule. Cells were centrifuged and the pellets re-suspended as before in 4 mL TS buffer. Following another centrifugation, cells were re-suspended in 0.6-mL saline, to which 1.2 mL sucrose-RNase-lysozyme solution was added (a stock solution contained 2.0 mL of 1 mg/mL boiled RNase, 44 mg lysozyme, 8.6 gm RNase-free sucrose, 19.0 mL TES4 buffer [0.05 M each of NaCl, ethylenediamine tetraacetic acid, and Tris, pH 8]). This suspension was incubated at 37 °C for 15 min, then at 55-60 °C for 3 min. To this solution was added (with gentle swirling) 0.6 mL 3.5% Sarkosyl (Sigma) in TES4, followed by a 20-min incubation at 55-60 °C. Pronase (Sigma) solution was prepared at 9 mg/mL in TES4 buffer and incubated at 37 °C for 60 min (autodigestion). An aliquot (0.25 mL) of this solution was added to the lysate followed by an overnight incubation at 37°C. Two phenol/chloroform extractions were performed by adding 1-mL water, 2.5-mL water-saturated phenol and 1.25-mL chloroform to the lysate, shaking gently and incubating on ice for at least 30 min prior to centrifugation at 5000 x "g" at 4 °C for 15 min. Following the second extraction, the aqueous layer was removed and extracted with 1.25 mL chloroform only. Following centrifugation of the chloroform extract, the top (aqueous) layer was removed and 1.5 volumes of ice-cold isopropanol were added. This mixture was inverted gently to precipitate the DNA. Precipitated genomic DNA was removed with a bent glass pipet, washed in ice-cold 100% ethanol, dried briefly, and dissolved in 1 mL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) in a sterile tube. The DNA concentrations were estimated based on comparisons with known standards in an agarose gel electrophoresis.

Table 1. *B. mallei* Strains Used in this Study

| Isolate name (this study) | Other Names | Source | Place of origin | Date | Isolated From |
|---|---|---|---|---|---|
| GB3 | 120, Strain A | Lister Institute, London, then USAMRIID | UK | 1920 | Unknown |
| GB4 | 10248, Strain 6 | USAMRIID | Ankara, Turkey | 1950 | Human |
| GB5 | 10229, Strain Budapest | USAMRIID | Pecs, Hungary | 1961 | Unknown |
| GB6 | 10260, Strain 11 | USAMRIID | Ankara, Turkey | 1949 | Human |
| GB7 | 10247, Strain 12 | USAMRIID | Ankara, Turkey | 1960 | Unknown |
| GB8 | Strain China 7 | USAMRIID | Unknown | 1942 | Horse |
| GB9 | Strain 102 | Imperial Inst. Vet. Res., then USAMRIID | India | 1932 | Mule lung |
| GB10 | Strain 106 | Imperial Inst. Vet. Res., then USAMRIID | India | 1932 | Horse |
| GB12 | Ivan, NCTC 10230 | USAMRIID | Pecs, Hungary | 1961 | Unknown |
| T2 | | Dr. David Miller at USDA-APHIS. Received from Etlik Veterinary Institute, Ankara, Turkey by Dr. Linda Schlater in 1984. | Turkey | | |
| T4 | | Dr. David Miller at USDA-APHIS. Received from Etlik Veterinary Institute, Ankara, Turkey by Dr. Linda Schlater in 1984. | Turkey | | |
| T6 | | Dr. David Miller at USDA-APHIS. Received from Etlik Veterinary Institute, Ankara, Turkey by Dr. Linda Schlater in 1984. | Turkey | | |

Table 1. *B. mallei* Strains Used in this Study (continued)

| Isolate name (this study) | Other Names | Source | Place of origin | Date | Isolated From |
|---|---|---|---|---|---|
| T7 | | Dr. David Miller at USDA-APHIS. Received from Etlik Veterinary Institute, Ankara, Turkey by Dr. Linda Schlater in 1984. | Turkey | | |
| T9 | | Dr. David Miller at USDA-APHIS. Received from Etlik Veterinary Institute, Ankara, Turkey by Dr. Linda Schlater in 1984. | Turkey | | |
| 273 | 2002721273 | CA Gleiser Army Med School | USA | 1956 | |
| 274 | 2002721274 | Ft. Detrick, then CDC | USA | 1956 | |
| 275 | NCTC 10245, GB11, 10399, China 5, 2002721275 | CDC | China | 1956 | Lung and nose of horse |
| 276 | 2002721276, G-2(3) | Naval Biological Lab, then CDC | Canada/USA | 1956 | |
| 277 | 2002721277, Kweiyang #4 | Gleiser Army Med Serv Grad School, then CDC | USA | 1956 | |
| 278 | 2002721278, 6317440 | CDC | NM/USA | 1964 | Human |
| 279 | 2002721279, A193 | CDC | NY/USA | 1964 | Human; from cord blood, nose, throat |
| 304 | 2000031304, 2000031281, H1533 | CDC | MD/USA | 2000 | Lab infection (human). Srinivasan et al., 2001. |
| 503 | 85-503 | Col. V.C. Micra, then USDA-APHIS | | | Equine |
| 567 | 86-567 | USDA-APHIS | East India | | Mule |
| ISU | | USDA-APHIS | ISU | | USDA-APHIS |

Abbreviations: USAMRIID = United States Army Medical Research Institute of Infectious Diseases, USDA-APHIS = United States Department of Agriculture Animal and Plant Inspection Service, CDC = United States Center for Disease Control and Prevention. Strains GB4-GB12 were received from Dr. Dave Waag at USAMRIID, strains Turkey 1 through ISU were received from Dr. David Miller at USDA-APHIS, strains 273 through 304 were received from Dr. Tanja Popovic at CDC. All abbreviations are listed as they were recorded when the isolates were received by ECBC.

## 2.3 DNA Analysis.

The DNA was digested with restriction enzymes according to the enzyme suppliers' recommendations. Restriction-digested DNA and $^{32}$P-labeled DNA molecular weight standards were size fractionated through a 20 cm long, 0.6% (w/v) agarose gel in 40 mM tris-acetate and 1 mM EDTA (TAE) at 30-60 V for 16 to 32 hr, according to the fragment sizes to be resolved and visualized by autoradiography. Southern transfer of gels to nylon membranes was performed according to Sambrook *et al.* [17] Molecular weight standards (1-12 Kb ladder from Invitrogen, Carlsbad, CA) and 1.5 to 48.5 Kb Lambda DNA mono cut mix from New England Biolabs (Beverly, MA) were labeled with [$^{32}$P]ATP). The 1-12 Kb ladder standards were labeled in a 20 µL reaction using 5-10 picomoles of 5' DNA ends, phosphorylation exchange buffer, and 5 µL of 3000 Ci/mmol, 10 mCi/mL gamma [$^{32}$P]ATP. Reactions were incubated at 37 °C for 30 min, inactivated by heating to 65 °C for 10 min, precipitated with 2.2 µL 3 M sodium acetate and 2.5 volumes cold 100% ethanol, stored at - 40 °C for 60 min, centrifuged, and washed twice with cold 70% ethanol, dried and re-suspended in TE buffer. For the Lambda standards, the same labeling was used following phosphatase treatment with 5 units of Antarctic Phosphatase (New England Biolabs) per microgram of DNA according to the enzyme manufacturer's recommendations.

For ribotyping experiments, genomic DNA was probed with a $^{32}$P-labeled 18-mer oligonucleotide. The oligomer was labeled using same method described above with the exception that the forward reaction buffer was substituted for the exchange buffer. The oligonucleotide sequence was derived from the *E. coli* rDNA gene sequence gct cct agt acg aga gga [18]. Hybridization was conducted overnight at 37°C in a solution containing 5X SSC (0.75 M NaCl, 0.075 M sodium citrate), 5X Denhardt's reagent and 0.5% (w/v) sodium dodecyl sulfate (SDS). Membranes were washed twice for 5 min each time in 2X SSC/0.1% (w/v) SDS at 50 C. Autoradiography was performed using Kodak cassettes for one to 10 days using Kodak Biomax MR film exposed at –80 °C.

## 3. RESULTS AND DISCUSSION

Six restriction enzymes were initially tested for their ability to produce RFLPs from B. mallei DNA. Digested DNA from nine isolates (GB3, GB4, GB5, GB6, GB7, GB8, GB9, GB10, and GB12) was probed with a labeled 18-mer probe derived from E. coli rDNA. Results confirmed that the labeled oligonucleotide derived from E. coli bound to at least 2-3 distinct bands of the B. mallei DNA, depending on the isolate. The restriction enzymes BamH1, ClaI, HindIII and SmaI yielded few observable polymorphisms, whereas EcoR1 and Pst1 single-enzyme digests yielded highly polymorphic patterns. Consequently, EcoR1 and Pst1 were selected for further study with the complete panel of isolates. The DNA was size-fractionated on agarose gels and hybridized fragments were sized by comparison with $^{32}$P-labeled commercial molecular weight standards in gels run for various times depending upon the size of the fragments to be resolved (typically 16-30 hr at 60V in a 20 cm gel). Southern-transferred DNA was probed initially with the rDNA oligomer, which yielded a polymorphic pattern of either two or three hybridized bands per isolate. Thirteen different EcoRI ribotypes were identified through the application of this method, and were designated E-1 through E-13.

Figure 1 shows the EcoR1 ribotype patterns of all 25 isolates (image is a composite of five different exposures). Table 2 shows the observed band sizes of all the hybridized fragments, as determined from electrophoretic size fractionations of various durations.

Hybridization with the rDNA oligomer was repeated with PstI-digested and fractionated DNA, and 12 different PstI ribotypes were identified (designated P-1 through P-12). Figure 2 shows the Pst1 ribotype patterns of all 25 isolates (image is a composite of two different exposures). The entire group of visualized Pst1 bands is shown in Table 2.

Together, the digests with EcoRI and PstI enzymes yielded a total of 17 distinct ribotypes from 25 isolates (Table 2). Ribotypes 5 and 9 contained four isolates each; groups 3 and 8 contained two isolates each, while the remaining groups contained only a single isolate.

## 4. CONCLUSIONS

Godoy *et al.* [19] conducted Multi Locus Sequence Typing (MLST) on five isolates of *B. mallei*, all of which are included in this study. No MLST-based sequence variation was observed among any of the *B. mallei* strains examined and the authors concluded that the *B. mallei* isolates represented a clone within the *B. pseudomallei* species. The polymorphisms revealed through ribotyping, and presented here, do not specifically address the relationship of *B. mallei* to other closely-related organisms, though they do provide an effective forensic means by which most of these isolates can be distinguished from each other.

Several interesting observations emerge from the ribotyping results. The first involves the relationship between GB8 and 304. Isolate 304 was obtained from a laboratory worker who became infected with GB8 (personal communication from Dr. Dave Waag).[20] In this study, the genotypic identity of GB8 and 304 and the clear discrimination of 304 from most of the rest of the isolates serves as a general illustration of the utility of this genotyping scheme for the discrimination of potential sources of an infection or outbreak involving *B. mallei*.

On the other hand, although the relationship between GB8 and 304 is clear, it is not apparent from available information whether or not the genotypically indistinguishable isolates 273 and 274 have any relationship with GB8 or 304 (although 273 and 274 were themselves collected in the same country in the same year). There are also no documented historical parallels between the genotypically indistinguishable isolates GB12 and 275, or GB5 and GB6.

Interestingly, four isolates collected in Turkey (T2, T4, T6, and T7) are all Ribotype 9. Isolate T9, collected in the same country, has a different ribotype (with the *Pst*I enzyme only). The similarity observed four of the Turkey isolates is suggestive of a common origin of these isolates.

These data suggest a practical genotyping approach such as that illustrated in Figure 1. Isolates would first be digested with *Eco*R1 and probed to determine their *Eco*R1 grouping. Isolates not adequately discriminated by their *Eco*R1 grouping would subsequently be digested with *Pst*1 to determine if they should be assigned the same or separate ribotype(s).

The same criteria used for discrimination of the 25 isolates from this study should be similarly useful for genotyping future isolates. If applied to more isolates and combined with more complete historical information, ribotyping may also elucidate the relationships among *B. mallei* strains with respect to geography and species. At a minimum, ribotyping is clearly useful for the forensic discrimination of *B. mallei* isolates that might be encountered in an outbreak.

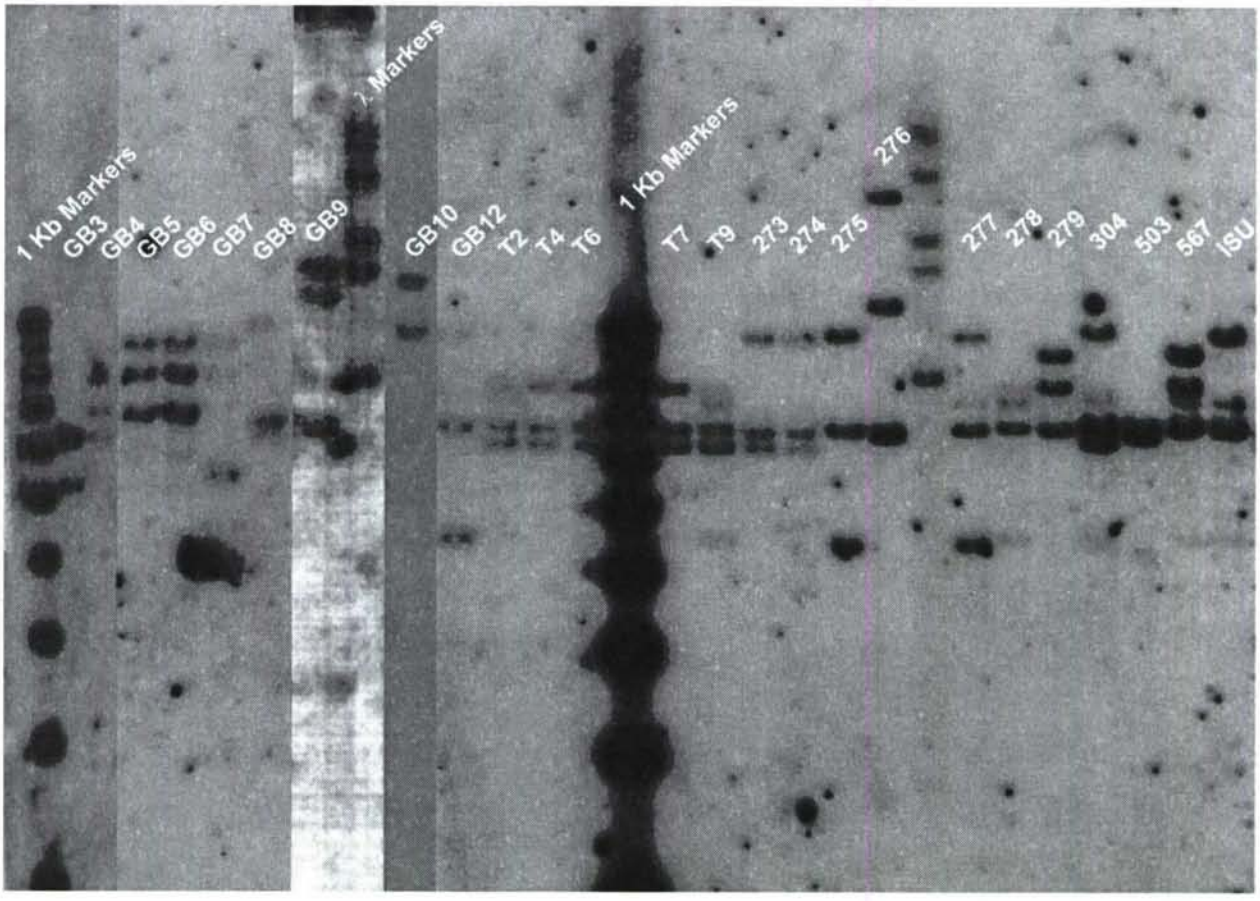


Figure 1. EcoR1 Ribotype Patterns

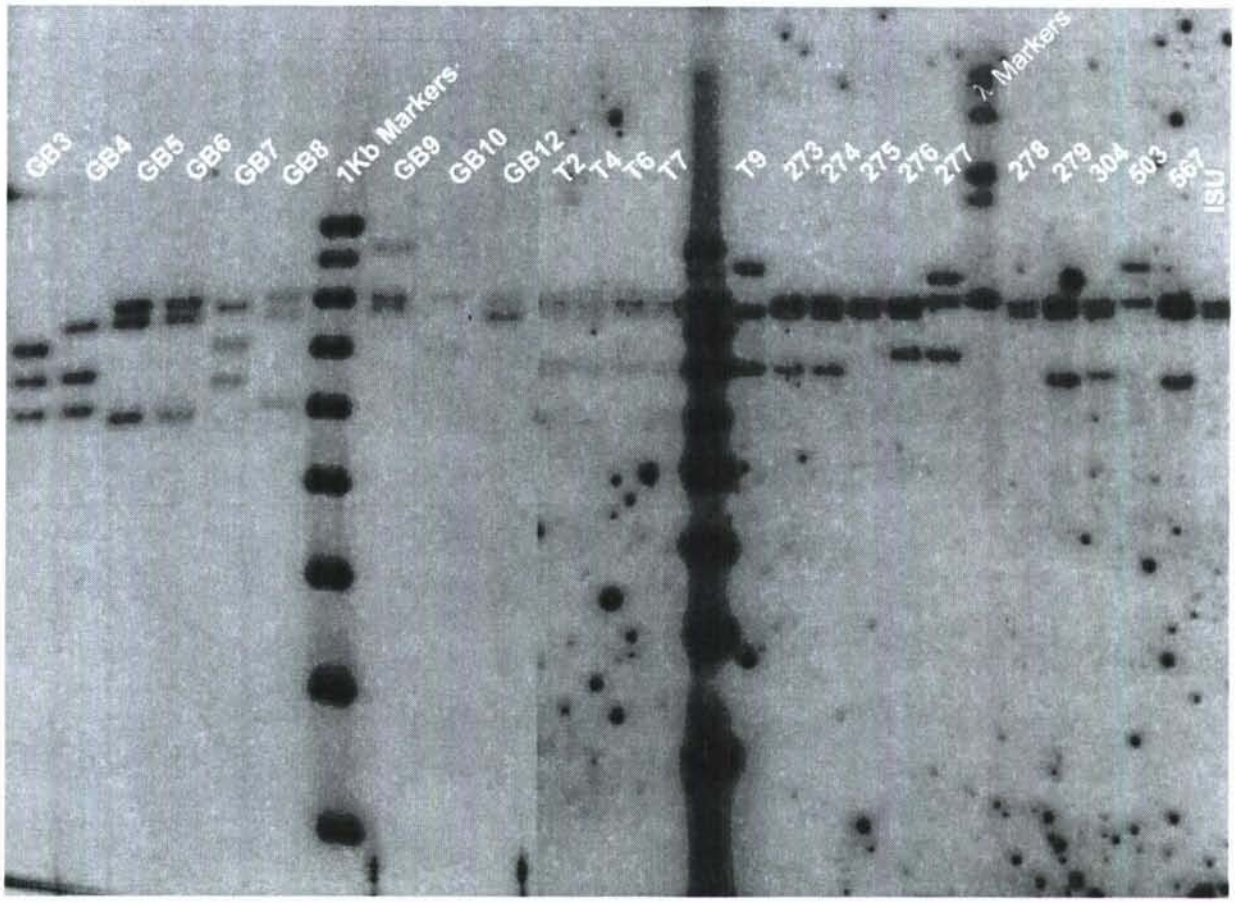


Figure 2. Pst1 Ribotype Patterns

Table 2. Ribotypes and Corresponding Autoradiography Bands from *Eco*R1 and *Pst*1 Digests

| **Ribotype** | **Isolates of this Ribotype** | ***Eco*R1 Group** | ***Eco*R1 Bands** | ***Pst*1 Group** | ***Pst*1 Bands** |
|---|---|---|---|---|---|
| 1 | GB3 | E-1 | 8.4, 7.2 | P-1 | 9.2, 8.6, 8.1 |
| 2 | GB4 | E-2 | 10.1, 9.2, 8.4 | P-2 | 9.9, 8.6, 8.1 |
| 3 | GB5, GB6 | E-3 | 11.2, 10.2, 9.0 | P-3 | 10.1, 9.8, 8.1 |
| 4 | GB7 | E-4 | 11.2, 10.2, 7.5 | P-4 | 10.0, 9.2, 8.5 |
| 5 | GB8, 273, 274, 304 | E-5 | 12.0, 8.8, 8.4 | P-5 | 10.1, 9.8, 8.1 |
| 6 | GB9 | E-6 | 15.0, 13.0, 8.8 | P-6 | 11.4, 10.1, 10.0 |
| 7 | GB10 | E-7 | 15.0, 11.2 | P-7 | 10.1, 9.2 |
| 8 | GB12, 275 | E-8 | 12.0, 8.8, 6.6 | P-8 | 10.1, 9.8 |
| 9 | T2, T4, T6, T7 | E-9 | 10.1, 8.8, 8.4 | P-5 | 10.1, 9.8, 8.1 |
| 10 | T9 | E-9 | 9.5, 8.8, 8.4 | P-9 | 11.4, 9.8, 8.1 |
| 11 | 276 | E-10 | 21.0, 13.0, 8.8 | P-10 | 10.0, 9.8, 8.4 |
| 12 | 277 | E-8 | 12.0, 8.8, 6.6 | P-11 | 10.9, 10.1, 8.4 |
| 13 | 278 | E-11 | 9.5, 8.8 | P-8 | 10.1, 9.8 |
| 14 | 279 | E-12 | 11.2, 10.2, 8.8 | P-5 | 10.1, 9.8, 8.1 |
| 15 | 503 | E-13 | 8.8, 8.4 | P-12 | 11.4, 10.1 |
| 16 | 567 | E-12 | 11.2, 10.2, 8.8 | P-3 | 10.1, 9.8, 8.1 |
| 17 | ISU | E-5 | 12.0, 8.8 | P-8 | 10.1, 9.8 |

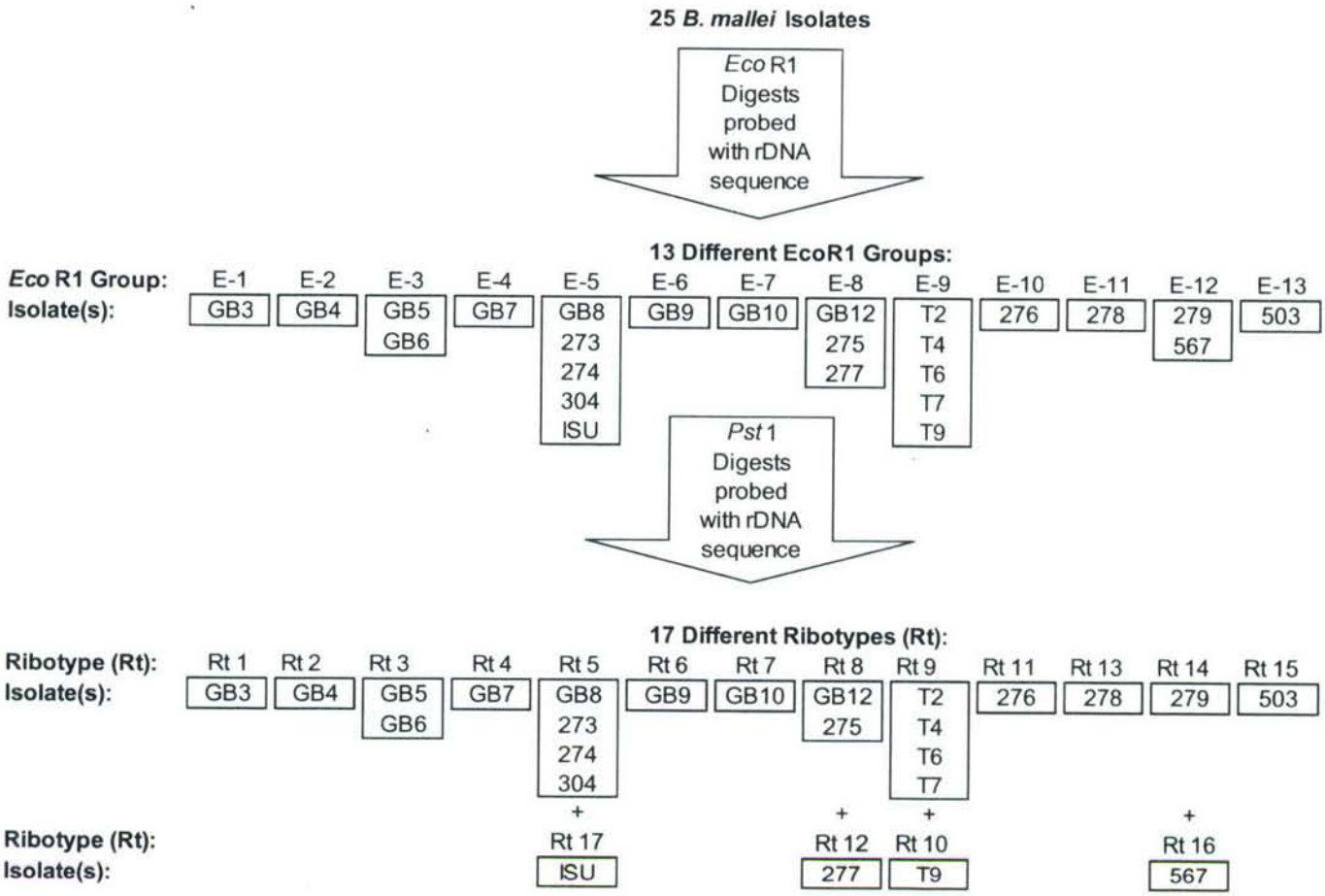


Figure 3. Hierarchical Scheme for Ribotype Discrimination of *B. mallei* Isolates. Isolates with indistinguishable *Eco*R1 groupings or indistinguishable ribotypes (Rt) are contained within the same box.

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